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Analysis of Fifteen Estrogen Metabolites Using Packed Column Supercritical Fluid Chromatography−Mass Spectrometry

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Packed column supercritical fluid chromatography with tandem mass spectrometry was used for the separation of estrone, estradiol, estriol, 16-epiestriol, 17-epiestriol, 16-ketoestradiol, 16α-hydroxyestrone, 2-methoxyestrone, 4-methoxyestrone, 2-hydroxyestrone-3-methyl ether, 2-methoxyestradiol, 4-methoxyestradiol, 2-hydroxyestrone, 4-hydroxyestrone, and 2-hydroxyestradiol. A gradient of methanol in carbon dioxide (0−30% methanol in 15 min, 2% change/min) at a flow rate of 2 mL/min and cyanopropyl silica column connected in series with a diol column, both 2.1 mm i.d. × 150 mm long, packed with 5-μm spherical silica-based particles, resulted in the separation and quantification of all 15 estrogens in less than 10 min. The limit of detection (LOD) and limit of quantitation (LOQ) of this pSFC MS/MS method was determined to be 0.5 (S/N = 3), and 5 pg, respectively. Compared with RP-HPLC MS analysis of the same mixture in terms of speed of analysis and sensitivity, pSFC MS is much faster, 10 versus 70 min, with comparable LOD and LOQ.

Estrone (E_1), estradiol (E_2), estriol (E_3), and their metabolites are a family of physiologically important compounds that have been linked to the possible development of breast cancer and other diseases.[1] The ability to separate and quantify a large number of estrogen metabolites in clinical samples is critical to determining the role each plays in cancer initiation, suppression, or both. To directly determine the role each metabolite plays in cancer requires analyzing hundreds, if not thousands, of population-derived clinical samples. This need accentuates the requirement that the analytical method be broad (i.e., to measure as many estrogen metabolites as possible), rapid (i.e., so that many samples can be analyzed quickly), and sensitive (in the pg range).

Despite this need, few studies have dealt with the separation and quantitative analysis of E_1, E_2, E_3, and their metabolites. A major reason for the lack of methods for the separation of a large number of estrogens simultaneously is the closely related structures of the estrogens, as is clear from Figure 1, which makes them difficult to separate. Current methods employed for the analysis of estrogen metabolites include radioimmunoassay,[2] enzyme immunoassay,[3] and high-performance liquid chromatography (HPLC) with electrochemical detection[4] or stable isotope dilution combined with analysis using gas chromatography−mass spectrometry.[5]

Unfortunately, these methods are either time-consuming, irreproducible, or lack sensitivity. The only procedure (to the best of our knowledge) that has shown the ability to resolve a large number of estrogens is HPLC with tandem mass spectrometry (MS/MS). Using this method, 15 estrogen metabolites can be completely resolved and quantitatively measured using stable-isotope dilution methods.[6] However, this method, which employs gradient elution, is time-consuming, requiring ~100 minutes/sample. Such a procedure would not be the method of choice for a study that requires the analysis of hundreds of samples if a faster method could be developed. Therefore, we explored the use of packed column supercritical fluid chromatography (pSFC) for the separation of estrogen metabolites. The analytical platform consisted of pSFC using carbon dioxide as the mobile phase and packed liquid chromatography columns as the stationary phase coupled on-line with MS detection for the direct analysis of estrogen metabolites.

Klesper et al. introduced SFC over 40 years ago as high-pressure gas chromatography above critical temperatures.[7] pSFC possesses many attributes common to HPLC such as the use of mobile-phase modification and gradient elution. However, pSFC analyses are faster than HPLC due to the lower viscosity and higher diffusivity of carbon dioxide, resulting in faster separations and higher efficiencies.[8] The mobile phase in HPLC is an organic solvent (normal phase) or water with an organic modifier (reversed phase). The mobile phase in SFC is carbon dioxide, a nonpolar solvent with a polarity similar to that of short-chain aliphatic hydrocarbons. Unlike water and organic solvents, carbon dioxide's

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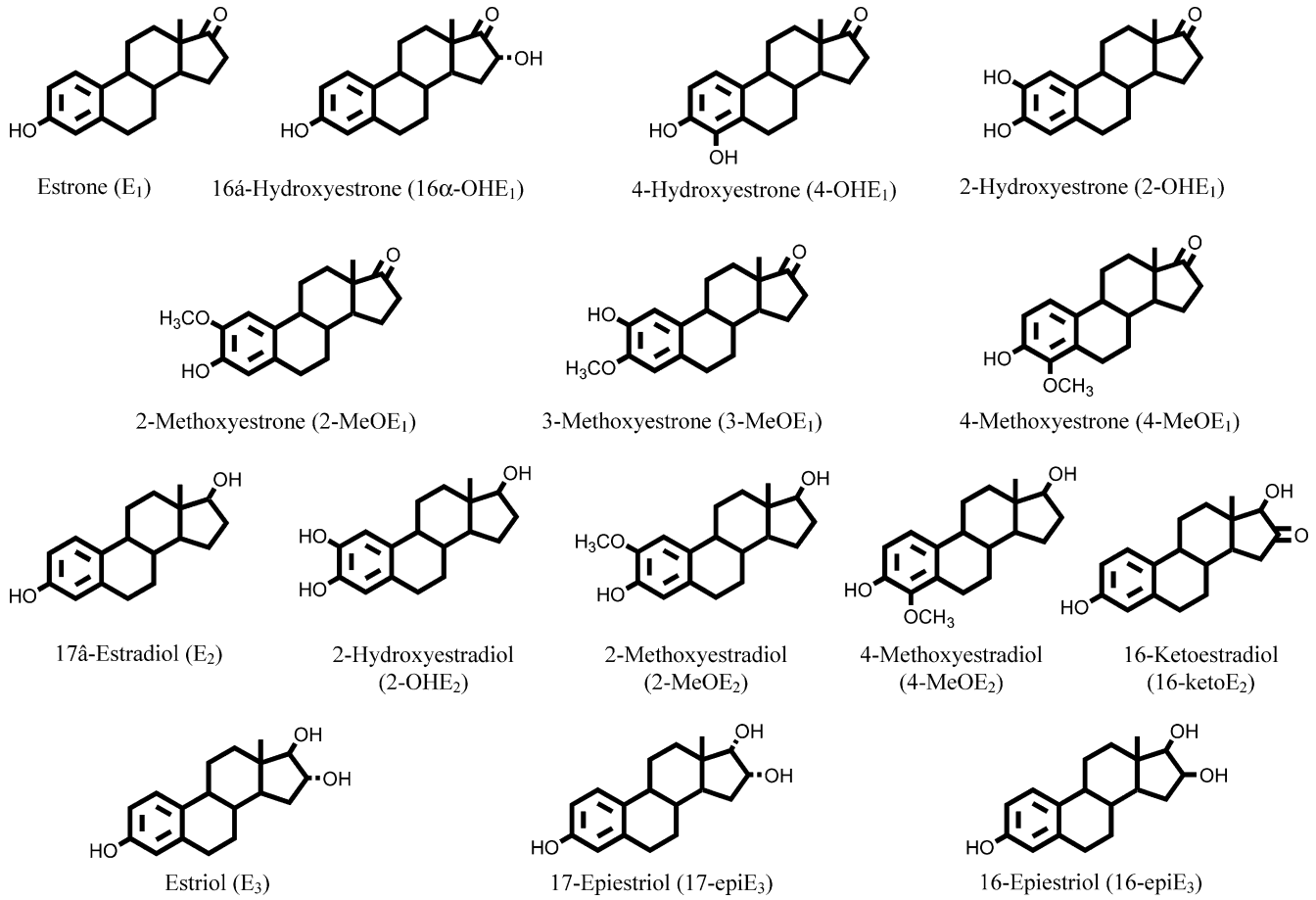

Figure 1. Structures of estrogen metabolites used in this study.

viscosity is more like that of a gas than a liquid, resulting in low back pressures even at high flow rates.[9] To increase the solvent strength and improve the separation resolution, modifiers such as polar organic solvents (methanol) or ammonia are commonly added to the carbon dioxide mobile phase.[8]

While there have been studies showing the separation of mixtures of E_1 and E_2 or E_2 and E_3,[10] there are none that show the use of SFC to separate large (i.e., >5) number of estrogens. The objective of this study is the evaluation and development of a fast and sensitive pSFC method for the analysis of 15 estrogens from an aqueous solution. The method should be fast (less than 15 min) with a sensitivity in the low-piocgram range. The results of pSFC−MS/MS will be compared to those obtained by HPLC−MS/MS analysis of the same group of estrogens in terms of speed and sensitivity. For the pSFC separation, two columns, a cyanopropyl CPS-2 hypersil (CPS) and a Betasil Diol-100 column will be evaluated separately and in series. A reversed-phase C-18 column will be used for the HPLC separation. Both techniques will employ mass spectrometry, not UV absorption, due to its sensitivity and universality as the method of detection and identification. The developed method would greatly aid in future analyses of clinical samples, urine, and serum for epidemiological studies designed to determine whether estrogen metabolite levels predict susceptibility to breast cancer.

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EXPERIMENTAL SECTION

Materials and Reagents. The estrogen metabolites (EM) E_1, E_2, E_3, 16-epiestriol (16-epiE_3), 17-epiestriol (17-epiE_3), 16-ketoestradiol (16-ketoE_2), 16α-hydroxyestrone (16α-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 4-methoxyestradiol (4-$MeOE_2$), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), and 2-hydroxyestradiol (2-OHE_2) were purchased from Steraloids, Inc. (Newport, RI). All estrogens and their metabolite standards have reported chemical and isotopic purity of ≥98% and were used without further purification. All other chemicals and solvents were reagent and HPLC grade, respectively.

Instrumental Setup. The supercritical fluid chromatograph (SFC) system was obtained from Berger SFC Analytix (Newark, DE). The instrument is equipped with an autosampler injector (LEAP Technologies, Cary, NC), a diode array UV detector (Agilent, Santa Clara, CA), and a mass spectrometer detector model ZQ MSD (Waters Corp., Milford, MA). All pSFC separations were acquired using MassLynx (Waters Corp.) and Massware (Mettler-Toledo, Newark, DE). The general SFC−MS conditions were as follows: source, atmospheric pressure chemical ionization (APCI); ion polarity, positive; multiplier, 450 V; desolvation and cone gas, nitrogen; desolvation gas, 400 L/min, cone gas, 28.7 L/min, APCI probe temperature, 500 °C; scan type, full scan 500−750 m/z.

Sensitivity determinations were made by injecting each of the 15 estrogen standards separately using LCQ Deca XP MS (ThermoFinnigan, San Jose, CA) in positive ionization mode with the

following parameters: ion source voltage, 5 kV; heated capillary temperature, 350 °C; capillary voltage, 7 V; sheath gas flow rate, 70 units; auxiliary gas flow rate, 15 units; tube lens offset, −15 V. MS full scan mode was employed for characterizing mass spectra of EM-Dansyl, whereas tandem MS full scan were used for quantitation. MS/MS full scan data for the protonated molecules [MH^+] of EM-Dansyl were obtained at a relative collision energy of 45% as follows: 2-OHE_1 and 4-OHE_1 m/z 753 → 125−800; 2-OHE_2 m/z 755 → 125−800; 16-ketoE$_2$ and 16α-OHE_1 m/z 520 → 125−600; E_3, 16-epiE_3, and 17-epiE_3 m/z 522 → 125−600; E_1 m/z 504 → 125−550; E_2 m/z 506 → 125−550; 3-$MeOE_1$, 2-$MeOE_1$, and 4-$MeOE_1$ m/z 534 → 125−600; 2-$MeOE_2$ and 4-$MeOE_2$ m/z 536 → 125−600.

Sample Preparation. The 15 estrogen metabolites were derivatized with dansyl chloride according to published procedures[6,11] in order to increase their ionization. The estrogen metabolites were dissolved in methanol containing 0.1% ascorbic acid and dried under a stream of nitrogen. The metabolites were resuspended in 100 μL of 0.1 M sodium bicarbonate buffer (pH 9.0) and 100 μL of dansyl chloride solution (1 mg/mL in acetone). After vortexing, the sample was heated at 60 °C for 5 min to form the dansyl derivatives. After derivatization, all samples were analyzed by pSFC electrospray ionization (ESI)-MS/MS.

pSFC Procedure. To determine the optimal column separation strategy for the resolution of the 15 estrogen metabolites, 20 μL aliqouts of the derivatized samples were separated using either a CPS or a Betasil Diol-100 column (both columns were gifts from ThermoElectron Corp., Bellafonte, PA). In addition, the same sample was analyzed using the two columns connected in series. Both columns had dimensions of 2.1 mm i.d. × 150 mm and were packed with 5-μm particles. The columns were maintained at a temperature of 35 °C during the analysis. To elute the metabolites, a methanol/carbon dioxide linear gradient of pure carbon dioxide to 30% methanol/carbon dioxide in 15 min (i.e., a change of 2% CH_3OH/minute) was used at a flow rate of 2 mL/min. The back pressure regulator was set to 100 bar. The effluent from the diode array detector was split upstream of the back pressure regulator using a Valco (Houston, TX) $^1/_{16}$-in. tee to direct a fraction of the flow to the mass spectrometer.

HPLC−ESI-MS^2. The analysis was performed using a Finnigan TSQ Quantum-AM triple quadrupole mass spectrometer coupled with a Surveyor HPLC system (ThermoFinnigan). Both were controlled by Xcalibur software (ThermoFinnigan). Liquid chromatography conditions: 150 mm long × 2.0 mm i.d. column packed with 4-μm Synergi Hydro-RP particles (Phenomenex, Torrance, CA) maintained at 40 °C; injection volume, 20 μL; mobile phase, a linear gradient changing the A/B solvent ratio from 72:28 to 85:18 in 75 min, solvent A and 0.1% (v/v) formic acid in water as solvent B. operating at a flow rate of 200 μL/min. The general MS conditions were as follows: source, ESI; ion polarity, positive; spray voltage, 4600 V; sheath and auxiliary gas, nitrogen; sheath gas pressure, 49 arbitrary units; auxiliary gas pressure, 23 arbitrary units; ion transfer capillary temperature, 350 °C; scan type, selected reaction monitoring (SRM); collision gas, argon; collision gas pressure, 1.5 mTorr. Collision-induced dissociation (CID) parameters for each estrogen metabolite are given in Table 1.

In addition, the following MS parameters were used for all experiments: scan width, 0.7 u; scan time, 0.50 s; Q1 peak width, 0.70 u fwhm; Q3 peak width, 0.70 u fwhm.

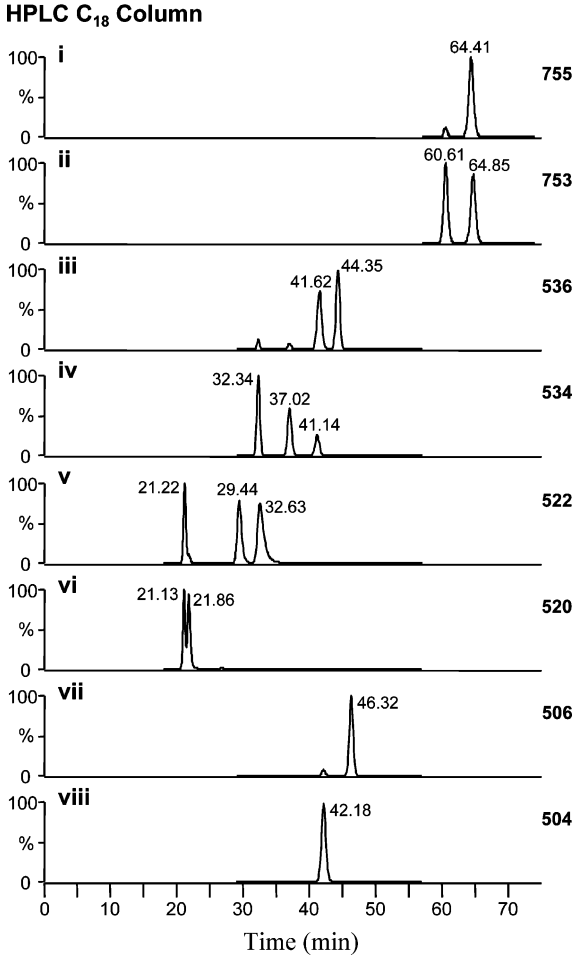

Figure 2. HPLC separation of the 15 estrogen metabolites using a reversed-phase C-18 column and tandem mass spectrometry. The m/z of the EM-Dansyl metabolites: E_1 504; E_2 506; E_3, 16-epi E_3, and 17-epi E_3 522; 16-ketoE$_2$, and 16α-OHE_1 520; 2-$MeOE_1$, 4-$MeOE_1$, and 3-$MeOE_1$ 534; 2-$MeOE_2$ and 4-$MeOE_2$ 536; 2-OHE_1 and 4-OHE_1 753; 2-OHE_2 755.

Table 1. CID MS Conditions for SRM of Each Estrogen

estrogen	m/z	monitor ion	collision energy (eV)
E_1	504	171	42
E_2	506	171	43
E_3	522	171	43
16-ketoE$_2$	520	171	43
16α-OHE_1	520	171	43
17-epiE_3	522	171	43
16-epiE_3	522	171	43
2-MeO E_1	534	171	42
3-MeO E_1	534	171	42
4-MeO E_1	534	171	42
2-MeO E_2	536	171	43
4-MeO E_2	536	171	43
2-OH E_1	753	170	44
4-OH E_1	753	170	44
2-OH E_2	755	170	43
d4- E_2	510	171	43
d3-E_3	525	171	43
2-OH E_1	525	171	43
d5−2-MeO E_2	541	171	43
d5−2-OH E_2	760	170	43

RESULTS AND DISCUSSION

A mixture of the estrogen metabolites shown in Figure 1 was prepared and used to test the ability of pSFC to resolve all 15

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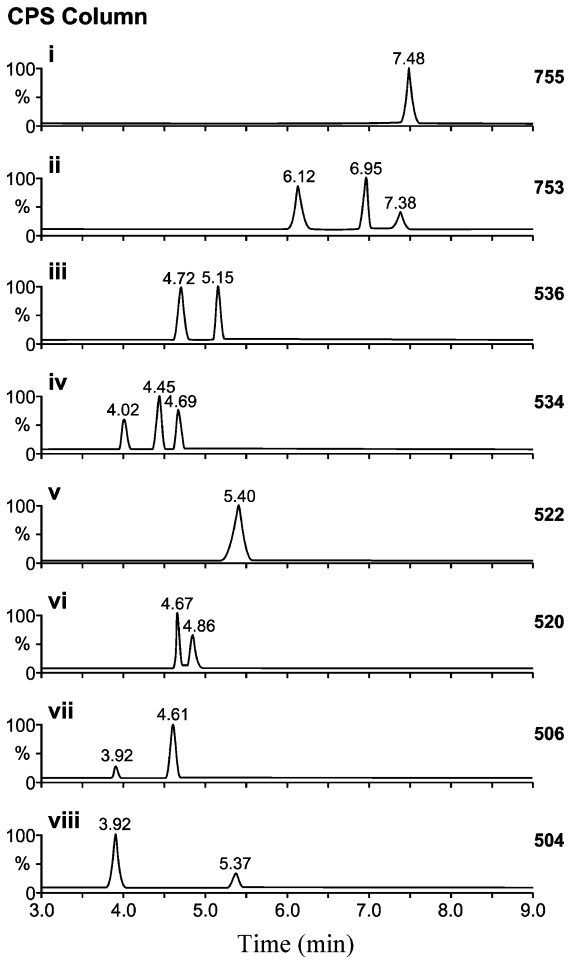

Figure 3. pSFC separation of the 15 estrogen metabolites using a cyanopropyl column and tandem mass spectrometry. Retention times are listed in Table 2.

Table 2. Division of the Fifteen Estrogens into Eight Groups According to Their *m/z* Values

group	*m/z*	estrogens
I	755	2-OHE_2
II	753	2-OHE_1, 4-OHE_1
III	736	2-$MeOE_2$, 4-$MeOE_2$
IV	534	3-$MeOE_1$, 2-$MeOE_1$, 4-$MeOE_1$
V	522	E_3, 16-epiE_3, 17-epiE_3
VI	520	16-ketoE_2, 16α-OHE_1
VII	506	E_2
VIII	504	E_1

compounds. While HPLC using UV detection is incapable of resolving all compounds in the mixture, single ion monitoring mass spectrometry allowed the resolution of all 15 estrogens metabolites (Figure 2). It is clear from Figure 2 that when the 15 estrogens were classified according to their *m/z* values, 8 separate groups resulted, Table 2. Members of each group were resolved and quantified by HPCL MS/MS. It was realized that, as in HPLC, the use of pSFC with UV detection would not result in the resolution of all 15 metabolites. Therefore, an approach similar to HPLC MS/MS using pSFC MS/MS was employed to resolve and quantify all 15 metabolites simultaneously based on the 8 groups, as mentioned above.

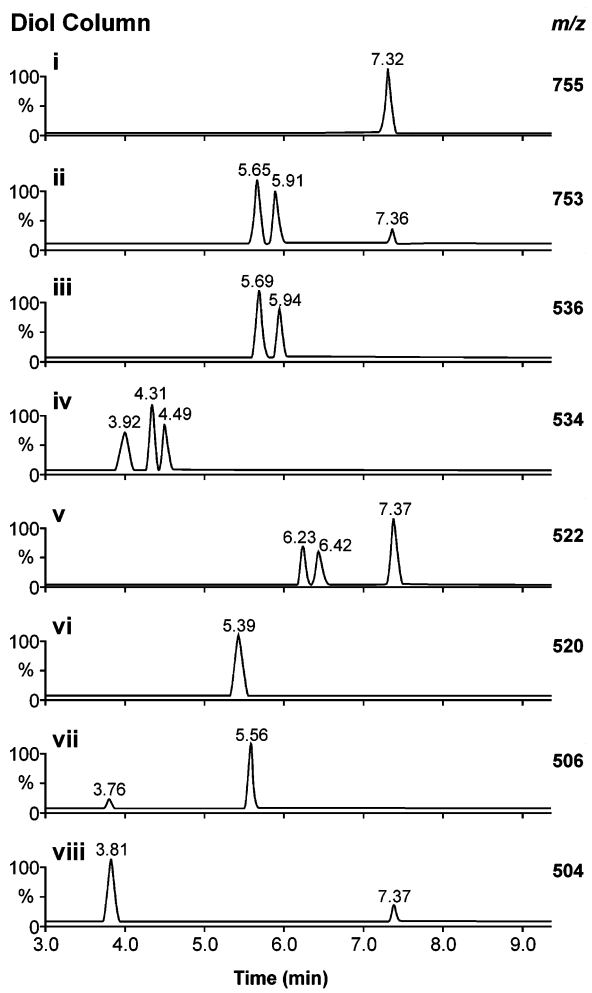

Figure 4. pSFC separation of the 15 estrogen metabolites using a diol column and tandem mass spectrometry. Retention times are listed in Table 2.

The initial attempts for the pSFC separation of the 15 estrogens using either a CPS or a diol column and modified carbon dioxide with methanol in an isocratic format was not satisfactory although different percentages of methanol were used at flow rates ranging from 1.5 to 4.0 mL/min.

Our initial attempt was to separate the 15 estrogen metabolites using a cyanopropyl column and a linear gradient of 100% CO_2 to 30% CH_3OH in CO_2 in 15 min (i.e., a change of 2% CH_3OH/min). Twelve of the 15 estrogens were completely resolved by SFC MS/MS over a period of 8 min, Figure 3. The only metabolites that could not be resolved were E_3 and its two epi-isomers, 16-epiestriol and 17-epiestriol.

pSFC employing a diol column was then used in an attempt to resolve all 15 estrogen metabolites. The column was operated with the same gradient conditions employed for the cyanopropyl column described above. Profile of the separation of the 15 estrogen metabolites is shown in Figure 4. Thirteen of the 15 estogrens were resolved in ~8 min, except for 16-ketoE_2 and 16α-OHE_1. Unlike the cyanopropyl column, however, the diol column was able to resolve E_3, 16-EpiE_3, and 17-EpiE3, Figure 5.

We hypothesized based on the elution order of the estrogens on the cyanopropyl and diol columns, which was different (Table 3), that connecting both columns in series and employing a gradient of CH_3OH in CO_2 may resolve all of the estrogen

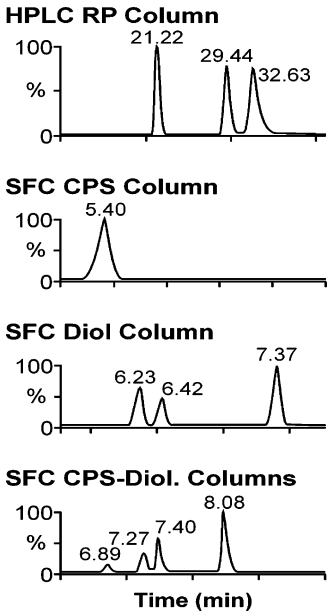

Figure 5. Comparison of the separation of E_3, 16-epi E_3, and 17-epi E_3 by reversed-phase HPLC, and pSFC using CPS column, diol column, and CPS-diol columns in series. Retention times are listed in Table 2.

Table 3. HPLC Retention Times vs pSFC Retention Times of the Fifteen Extrogens[a]

estrogen	LC-C18	pSFC-CPS	pSFC-diol	pSFC-CPS/diol
E_1	42.1	3.92	3.81	5.25
E_2	46.32	4.61	5.56	6.57
E_3	21.22	5.40	7.37	8.08
16α-OHE_1	21.86	4.67	5.39	6.47
16-ketoE$_2$	21.13	4.86	5.39	6.61
16-epiE_3	29.44	5.40	6.42	7.40
17-epiE_3	32.63	5.40	6.23	7.27
2-OHE_1	60.61	6.95	5.95	7.94
2-OHE_2	64.41	7.48	7.32	8.85
4-OHE_1	64.85	6.12	5.65	7.36
2-MeOE_1	37.02	4.45	4.31	5.72
2-MeOE_2	41.62	5.15	5.94	7.02
3-MeOE_1	32.34	4.69	4.49	5.95
4-MeOE_1	41.14	4.02	3.92	5.35
4-MeOE_2	44.35	4.72	5.69	6.69

[a] Retention times are expressed in minutes.

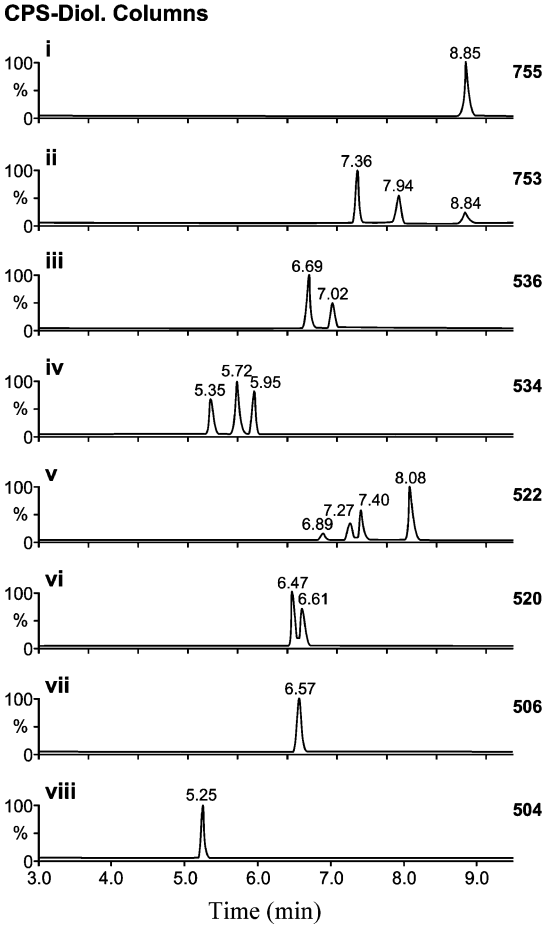

Figure 6. pSFC separation of the 15 estrogen metabolites using CPS-diol column in series and tandem mass spectrometry. The *m*/*z* of the EM-Dansyl metabolites are the same as in Figure 2. Retention times are listed in Table 2.

metabolites. The separation was configured using the cyanopropyl column first in the series. As shown in Figure 6, all 15 estrogens were completely resolved in less than 10 min.

Comparison of the elution times by pSFC using the CPS, diol, and CPS-diol columns in series (Figures 2, 3, and 5) revealed that the migration times by CPS-diol columns in series, albeit being higher than that of the CPS and diol columns, are not additive.

As mentioned earlier, one of our objectives was to develop a method that possesses the same or better sensitivity than the HPLC MS/MS method of 2 pg. Although all separations were obtained using the SFC instrument with the ZQ MSD, we realized that this single quadropole instrument did not give the required sensitivity, so it was decided to use the LCQ Deca MS instead of the ZQ MSD to determine the sensitivity of the method. With the LCQ Deca employing tandem mass spectrometry we achieved a sensitivity of 1pg, which is better by an order of magnitude than when ZQ MSD was used. The limit of detection and limit of quantitation of the pSFC MS/MS method using estrogen standards in 0.1 M bicarbonate buffer solution were determined to be 0.5 (S/N = 3) and 5 pg, respectively.

CONCLUSIONS

The objectives of this study were the evaluation of pSFC and the development of a rapid method for the broad analysis of estrogen metabolites without sacrificing the sensitivity achievable using HPLC MS/MS.[6] As shown in the results, the pSFC MS/MS analysis using a dual column system required less than 12 min to completely resolve all 15 estrogen metabolites while the analysis by HPLC MS/MS[6] required 100 min (including column regeneration and equilibration). Generally, both techniques when combined with mass spectrometry gave baseline resolution and allowed quantification of all the estrogens in the test mixture. While the SFC method is faster than HPLC, it had a comparable level of sensitivity (0.5 pg for pSFC and 1 pg on-column for HPLC). Examining the peak widths obtained by each technique reveals that the baseline width in pSFC is in seconds (~20−30 s) while in HPLC is in minutes (~1.8 min), which contributes to higher resolution and faster analysis times.

These results, employing a simple methanol/carbon dioxide linear gradient to separate and quantify all 15 estrogens in less

than 10 min, reveal the power of pSFC MS/MS in resolving and quantifying closely related (structure) estrogens that differ by the group (proton, methoxy, hydroxy, or keto) or position of substitution on the ring. For example, 16α-hydroxyestrone and 16-ketoestradiol, which have closely related structures (Figure 1) and the same m/z values, were resolved from each other. Also, it is interesting to note that estriol and its two 17-epiestriol and 16-epiestriol, which have the same groups occupying the same positions on the ring structure, except for their position into- or out-of-the plane, were baseline resolved and easily quantified. Also, the three methoxyestrone isomers were baseline resolved.

The developed pSFC method will be tested for the analysis of estrogen metabolites from urine and serum, and the results will be published in a future report.

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